"Aplicaciones de la Citometría de Flujo en la práctica clínica"

Separación celular por Citometría de Flujo: *cell SORTING*

Plácida Baz

Farmacéutica y Bioquímica

Citometría de Flujo

Laboratorio Inmunogenética

INIGEM-CONICET-UBA

Cell SORTING

Definición:

El cell Sorting es un método de separación y purificación de células, identificadas mediante varios parámetros, a partir de una muestra heterogénea.

Posteriormente pueden ser utilizadas con diversos fines, ya sea diagnósticos, terapéuticos o en investigación.

Temario:

- Fundamento
- Aplicaciones
- Técnicas y Requisitos
- Control de Calidad
- Ejemplos
- Bibliografía

Fundamento

Métodos de Separación celular

- **<u>Un solo parámetro de clasificación</u>:**

  No producen alta tasa de purificación

  ENRIQUECIMIENTO

- **<u>Varios parámetros de clasificación</u>:**

  Buena Recuperación y Rendimiento

  Alta Pureza

  Rápido

  Mantienen Viabilidad y Funcionalidad celular

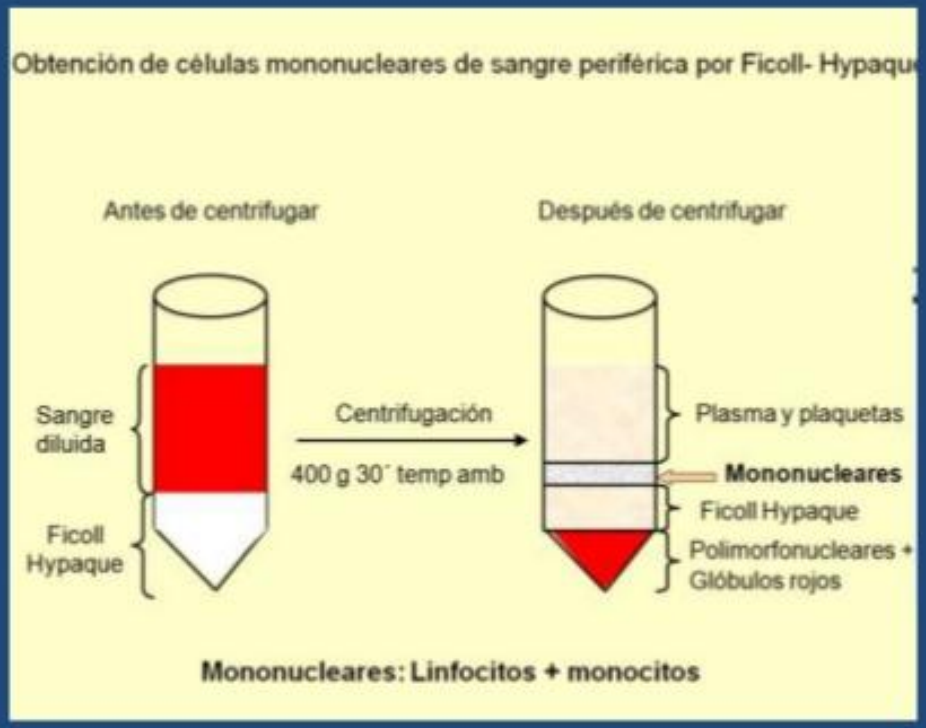
Criterios de Selección celular

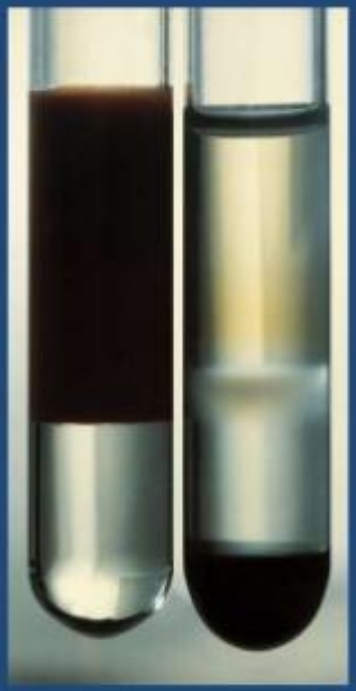
Seleccionamos las células de interés basados en los siguientes parámetros:

- **Tamaño**
- **Densidad celular (Gradiente de Ficoll-Hypaque)**
- **Adherencia diferencial (al Plástico: Monocitos y Macrófagos)**
- **Afinidad Ligando – Receptor: a) Rosetas: Linfocitos T**
  b) Columnas Inmunomagnéticas
- **Inmunofenotipo (Citometría de Flujo)**

Enriquecimiento previo

TÉCNICA FICOLL-HYPAQUE





Sorting por Citometría de Flujo

- **Se identifica la subpoblación celular a separar basado en varios criterios de clasificación.**
- **Tamaño y granularidad**
- **Expresión antigénica**
- **Marcación inmunocitométrica policromática**

Aplicaciones

Aplicaciones Sorting por Citometría

- **Cultivo celular**: Clonación célula única (Hibridoma AcMo)
  Stem cells (células progenitoras pluripotenciales)
  Células Transfectadas con gen Reportero (GFP)
  Estudios de Funcionalidad
- **Estudios genómicos**: Extracción ADN (FISH)
  Extracción ARN (Microarrays / RT-PCR)
- **Terapeúticos**: LT en Enfermedad injerto contra Huésped
- **Sexado**: Reproducción (Crom X/Y por contenido ADN)
- **Industria Farmacéutica**: Mecanismo de acción de Drogas
- **Microscopía**: Estudio estructuras celulares (Portaobjetos)

Técnicas

Técnicas de Sorting por CMF

- **Mecánica**: a) Tubo intercalante
  b) Pulso Acústico (ATTUNE ®)

- **Deflección Electrostática**: Flujo en Aire

Sorting Electrostático vs Mecánico

Table 1. Electrostatic vs. Mechanical Sorting

Electrostatic (Stream in Air)	Mechanical
Sorts up to 100,000 events/second	Processes up to 10,000 events/sec but only sorts up to 300 events/sec
Produces droplets (aerosols)	Fully contained
Prone to blockages	No nozzle to block
At least two sorts – four possible	Only one sort
Sorts at a reasonable concentration	Usually requires a "concentrator"
Multiple receptors available (tubes/slides/multiwell plates)	Not applicable

Sorting de Flujo Mecánico

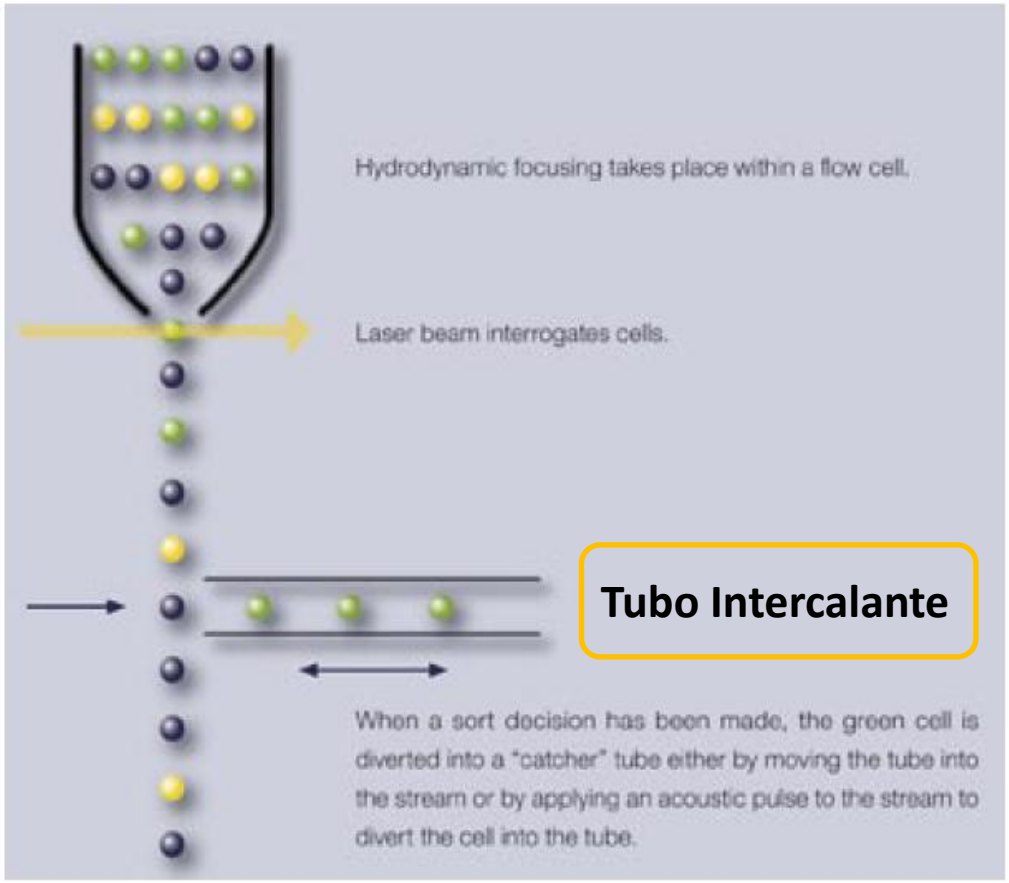
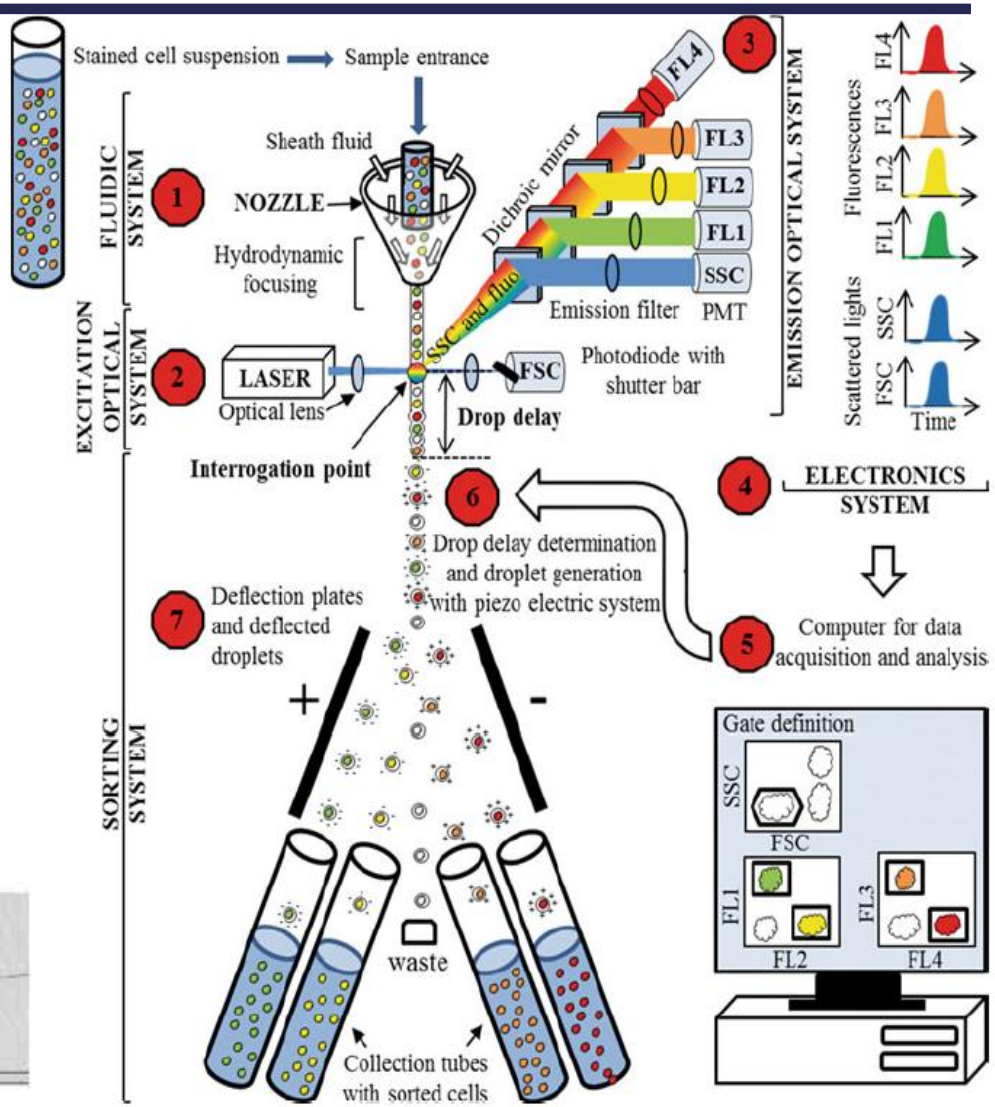

Hydrodynamic focusing takes place within a flow cell.
Laser beam interrogates cells.
Tubo Intercalante
When a sort decision has been made, the green cell is diverted into a "catcher" tube either by moving the tube into the stream or by applying an acoustic pulse to the stream to divert the cell into the tube.

Figure 2. Mechanical Sorting

BD FACScalibur

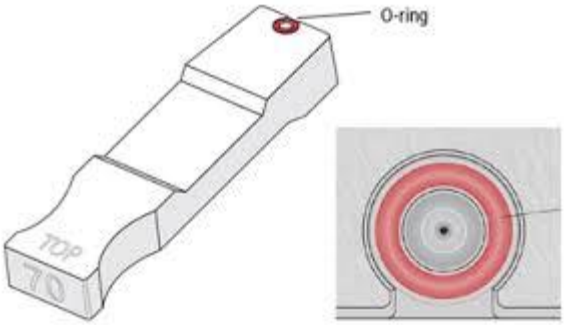
Deflección Electrostática

Fig. 1 Flow cytometry principle

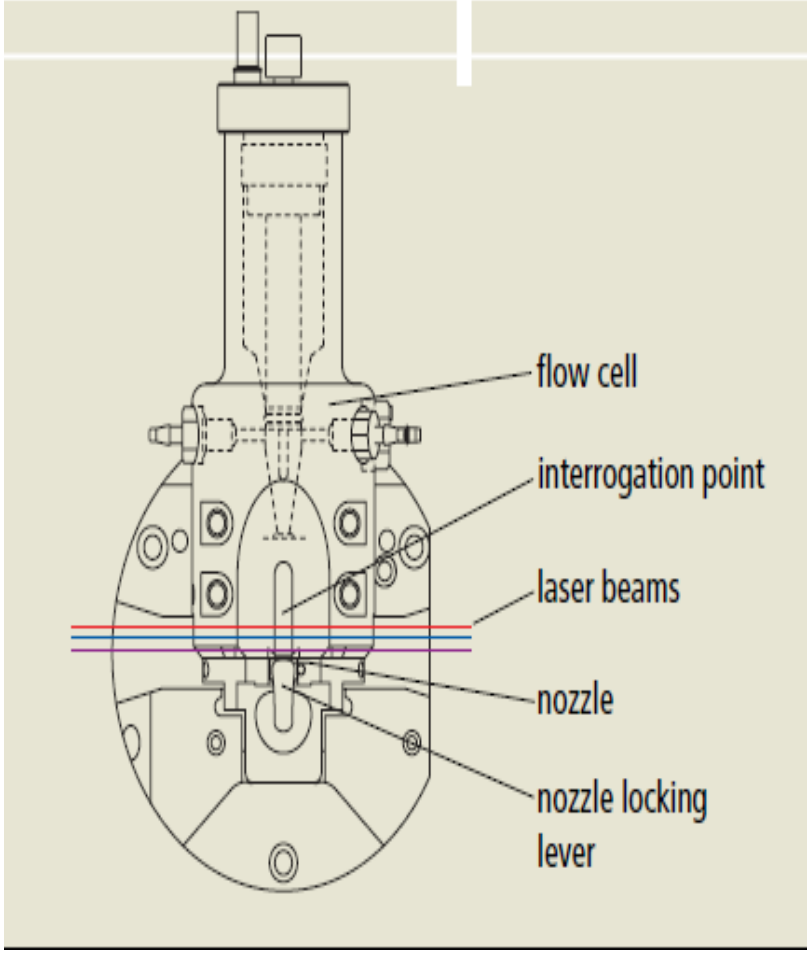


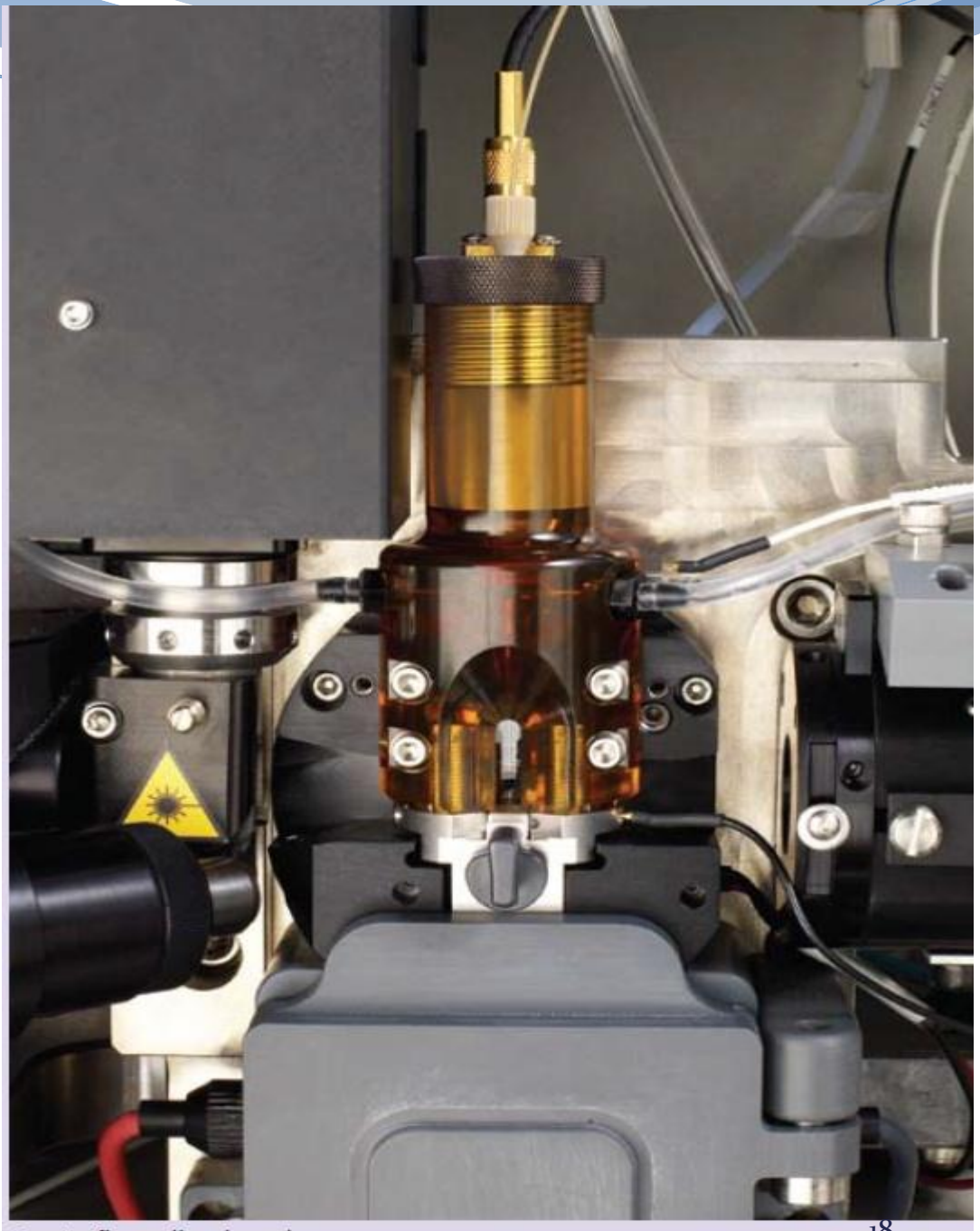
Flujo en Aire

NOZZLE



BD FACSAria II

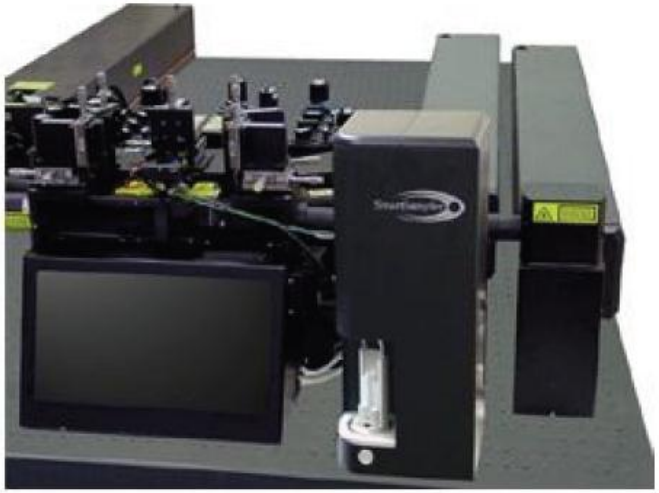




Cuvette flow cell and nozzle

BD VantageSE DiVa option

Beckman Coulter Epics Altra Hypersort



Beckman Coulter MoFlo,
MoFlo Astrio

BD FACSAria

BD FACSAria Fusion: Área Estéril

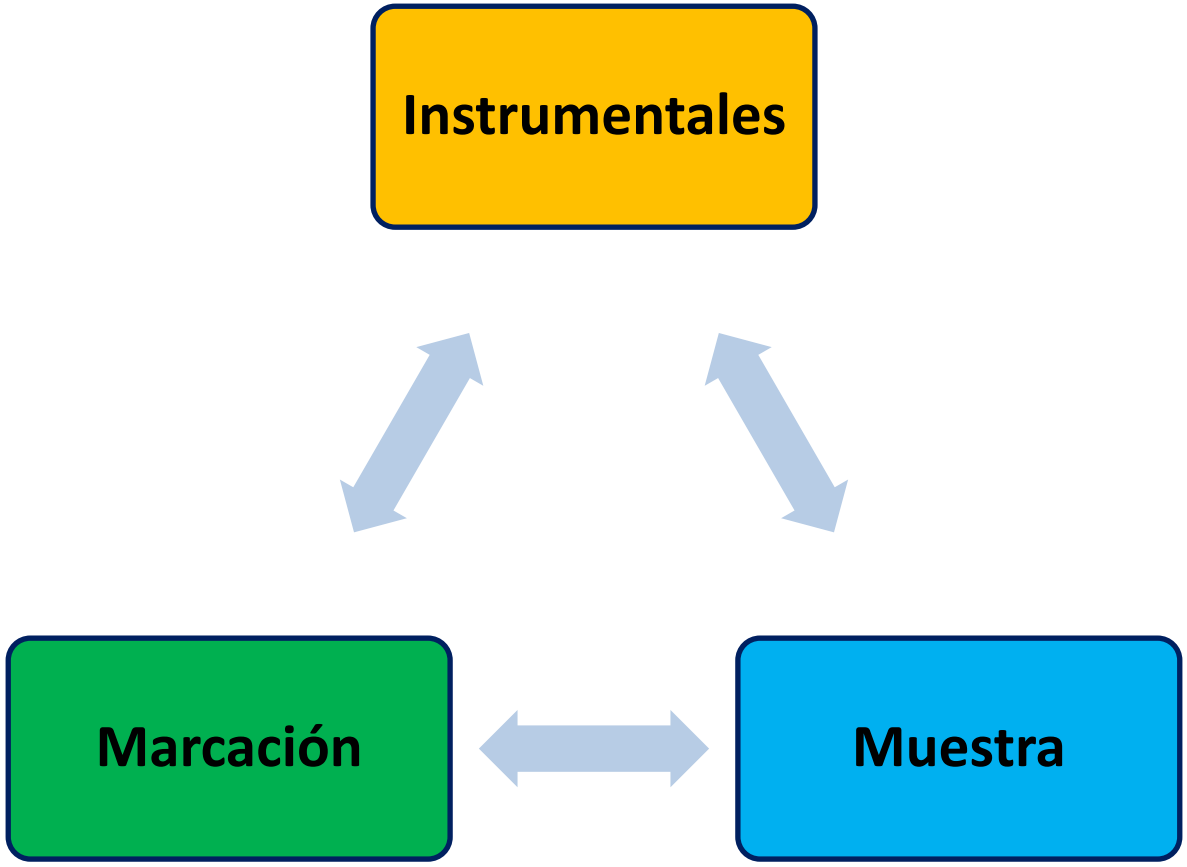
BIOSEGURIDAD

Recomendaciones Bioseguridad

- **Manipulación de la Muestra**
- **Entrenamiento del Personal**
- **Medidas de Protección Personal**
- **Descontaminación y puesta a punto del Citómetro**
- **Eficiencia en el control de liberación de Aerosoles**

Requisitos

Sorting por Citometría de Flujo



Planificación:

- Plantear nuestra Pregunta
- Decidir si la Citometría de Flujo pueda responderla
- Evaluar cuál es nuestra grupo poblacional (especie)
- Qué células, tejidos, líneas celulares
- Cuántas poblaciones necesitamos separar
- Cuál es su Inmunofenotipo

Fase previa

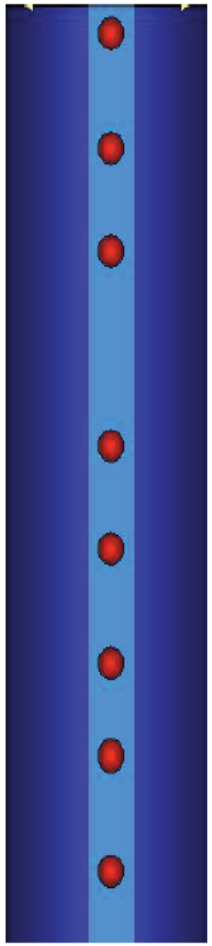
- **Armado de Panel**
- **Marcación citométrica**
- **% de la población en nuestra muestra a Sortear**
- **Calcular la cantidad de células a marcar**
- **Enriquecimiento previo**
- **Decidir condiciones de Sorting (Aséptico ó No)**

Nozzles Recomendados: FACS Aria II / III

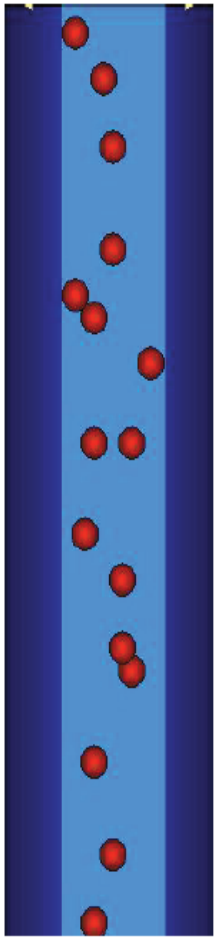
Nozzle (µm)	Presión (psi)	Frecuencia gotas/seg	Tamaño celular	Concentración (cels/ml)
70	70	90	LT sin activ / LB Plaquetas	10 millones
85	45	50	LT activ/ CP/NK M/DC/Stem cells	8 millones
100	20	20	Neurons/Macrof Lin celulares	6 millones
130	10	10	Líneas >15µm Céls Vegetales	4 millones

El diámetro del orificio del Nozzle debe ser 5 veces mayor al celular

La velocidad de *sorting* es un equilibrio entre la concentración de la muestra y la presión diferencial



- $7(3\text{-}5)x10^7$ células/ml
- 20.000 eps
- 0.3 psi (15µl/Min.)
- Tiempo de sorting ~1h
- Corriente central estrecha
- Pocos dobletes y coincidencias
- Buena recuperación y pureza



- $5x10^6$ células/ml
- 0.7 psi (60µl/Min.)
- 5.000 eps
- ~5.5h para 10^8 células
- Corriente central ancha
- Muchos dobletes y coincidencias
- Baja pureza y recuperación

Control de calidad

(*)Óptica: Fuente de iluminación, detectores, separación espectral

(*)Electrónica: análisis de pulso, control de los detectores, conversión digital

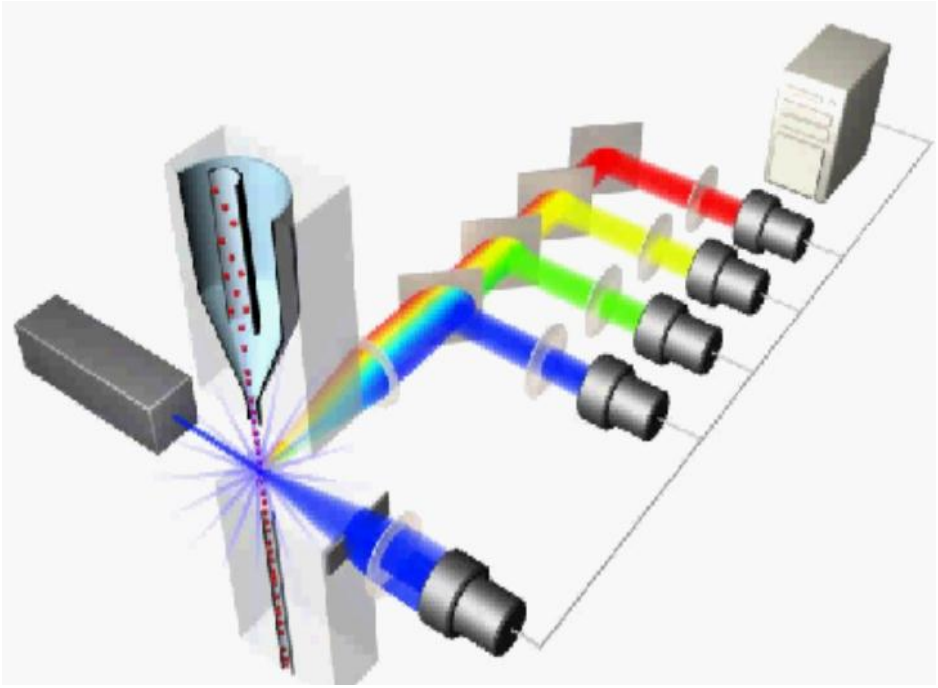
Fluídica: hidroenfocado, presiones, niveles de Fluidos, LIMPIEZA!!!

Adquisición: velocidad, filtrado de muestras, número de eventos

Análisis de datos: Presentación, análisis, cuantificación de poblaciones

BIOSEGURIDAD

(*) LLAMAR al Servicio Técnico



Previos al Sorting

- **CS&T**
- **Estabilizar el Flujo y Break Off**
- **Side Stream**
- **Drop Delay (Accudrop®)**

CS&T®

BD

BD™ CS&T Beads

Catalog No.	Tests
656504	50
656505	150

4/2016 23-14666-01

IVD CE

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1. INTENDED USE

BD™ CS&T beads are used on a BD flow cytometer to provide a standardized method to perform quality control of the instrument's optics, electronics, and fluidics, and for adjusting fluorescence compensation. On some BD instruments, BD CS&T beads are also used for adjusting detector voltages.

2. SUMMARY AND EXPLANATION

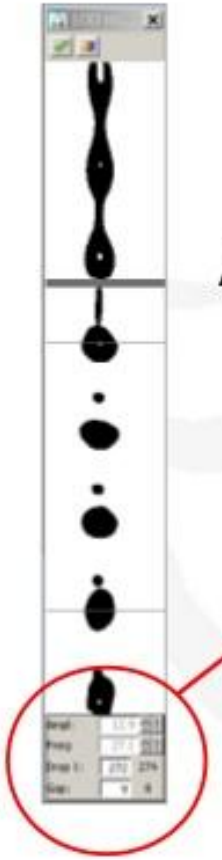
BD CS&T beads are a suspension of fluorospheres with uniform and stable size and fluorescence intensity. The beads are used for instrument quality control (QC) to characterize, track, and report performance measurements of supported flow cytometers. The cytometer's software displays current bead data in plots. Forward scatter (FSC) and side scatter (SSC) identify bead populations based on relative size.

The beads enable the software to measure detector performance and are used to measure the sensitivity of each fluorescence detector. Sensitivity is a measure of the cytometer's ability to resolve dimly stained cells. In addition, the beads are used to optimize the compensation settings each time instrument QC is run.

The software calculates the bright bead median, bright beads %rCV (robust coefficient of variation), and instrument sensitivity for FSC, SSC, and each fluorescence parameter, and compares them to expected values for the bead lot. The rCV measures cytometer alignment.

Daily measurements are automatically entered into Levey-Jennings plots. This allows you to monitor instrument

Ajuste del Break Off y Formación de Gotas



Droplet Creation

Aria – 100um

Settings

Ampl = Amplitude (how hard the stream is being vibrated)

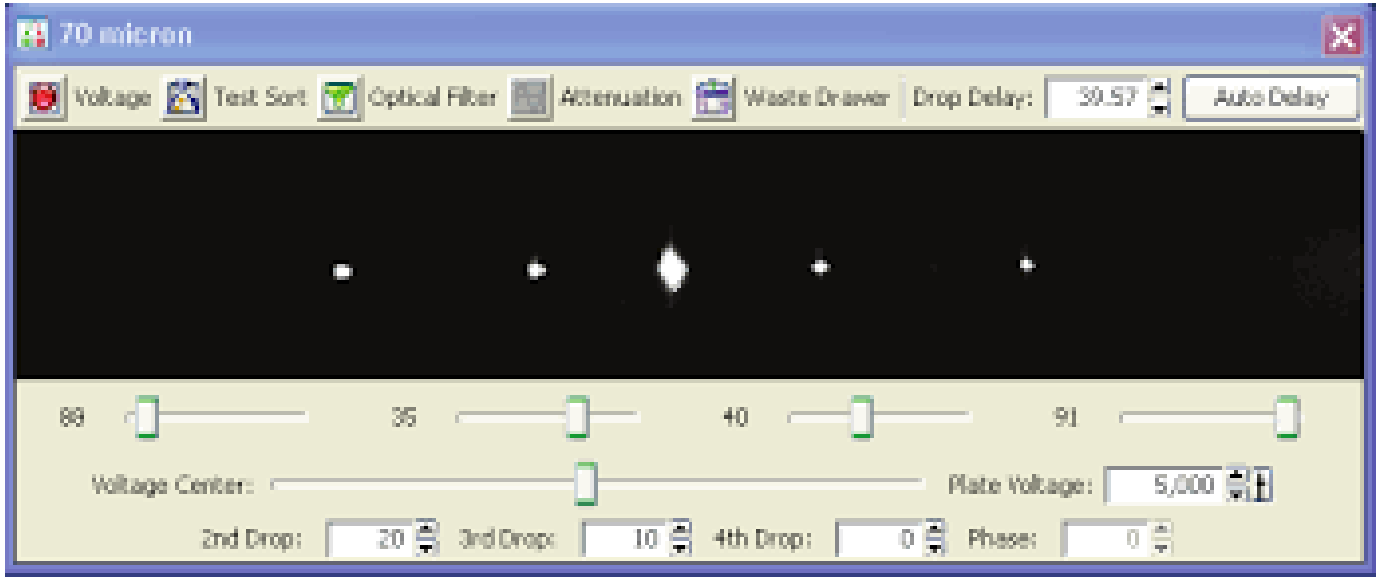
Freq = Frequency (how many droplets are being created per second – in KHz)

Drop 1 = Arbitrary point of first separated drop (measures in pixels but must be a known time offset from the trigger)

Gap = distance between drops (in Pixels) – used for stream monitoring

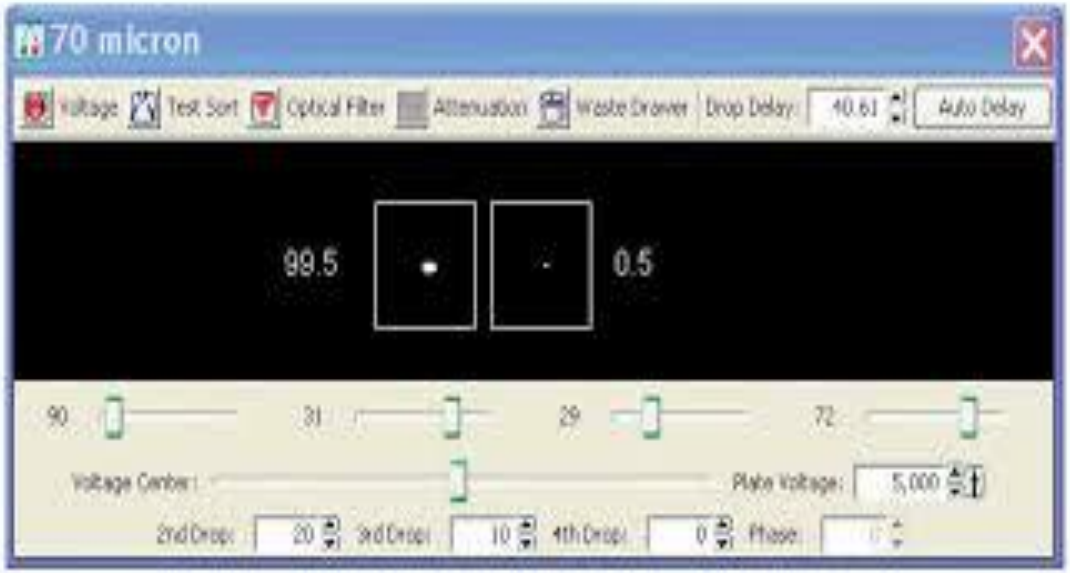
<u>Condición ideal de Sorting</u> : Una sola célula por gota

Side Stream:


70 micron
Voltage
Test Sort
Optical Filter
Attenuation
Waste Drawer
Drop Delay:
39.57
Auto Delay
89
35
40
91
Voltage Center:
Plate Voltage:
5,000
2nd Drop:
20
3rd Drop:
10
4th Drop:
0
Phase:
0

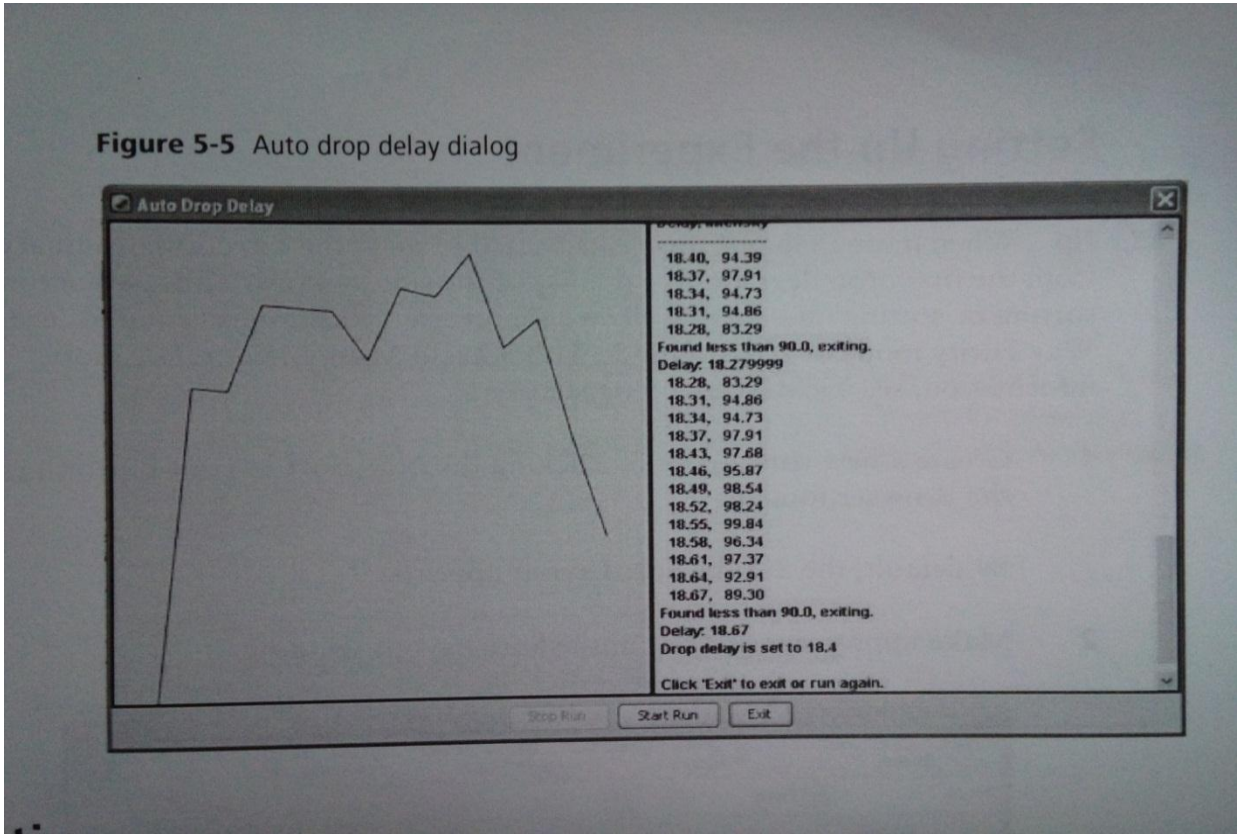
Deflección de los chorros

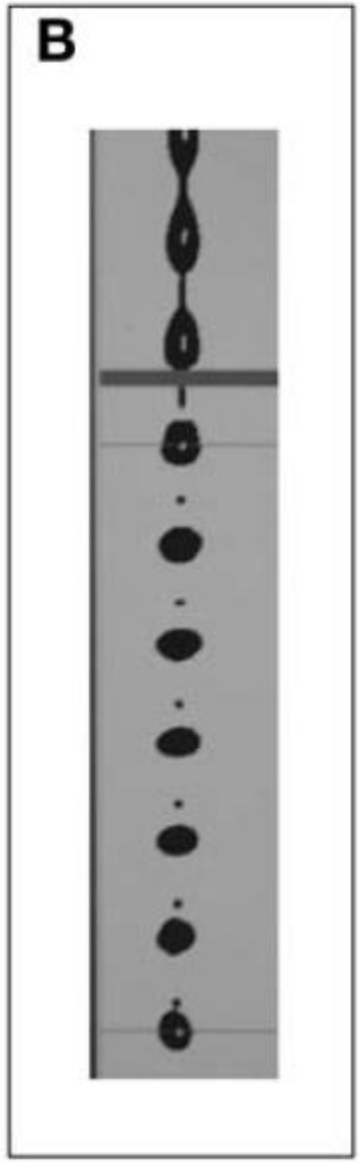
Drop Delay: Accudrop®



Ajuste de la carga eléctrica de la gota a separar

Ajuste del Drop Delay





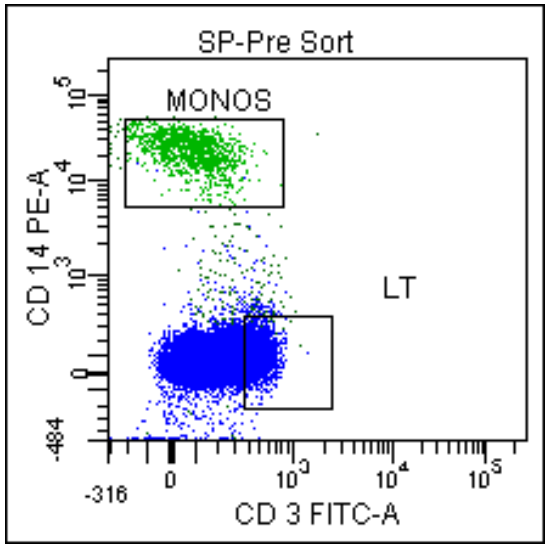
Accudrops ® catalog No.661612

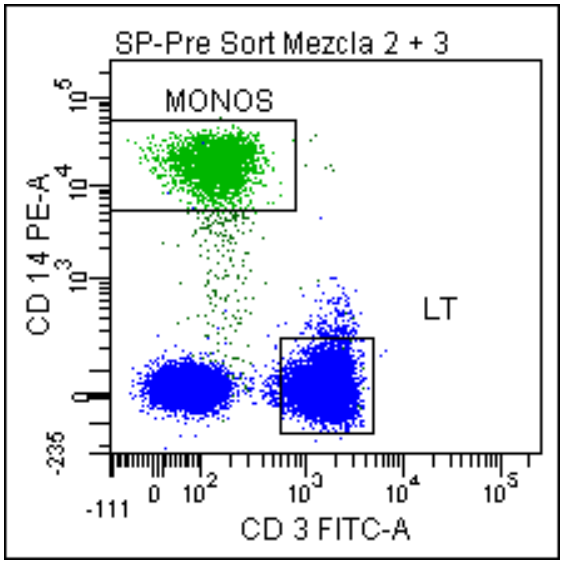
Durante el Sorting

- **Threshold y Area Scaling**
- **Exclusión de Dobletes y células muertas**
- **Compensación**
- **Sort Lay-Out**
- **Eficiencia de Separación**

Muestra para SORTING

COMPENSACIÓN





En el primer tubo se uso menor cantidad de Ac aCD3 => hubo menor separación entre las 2 poblaciones

En la mezcla 2+3 se agregó AcMo y por lo tanto se pueden diferenciar las poblaciones (+) de la (-)

TITULAR AcMo

BD™ CompBead and BD™ CompBead Plus Compensation Particles

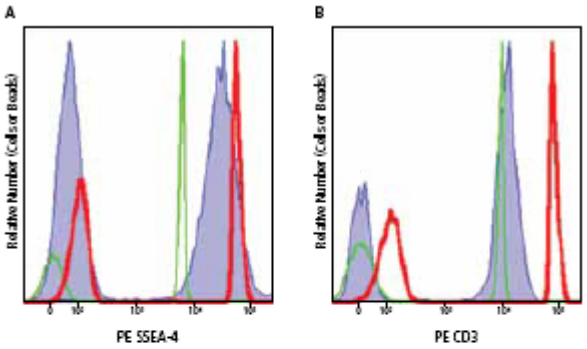


Figure 1. Comparison of BD CompBead and BD CompBead Plus particles on different sample types.

A. H9 human embryonic stem cells (WiCell, Madison, WI), and BD CompBead (Cat. No. 552843) and BD CompBead Plus particles (Cat. No. 560497) were stained with PE mouse anti-SSEA-4 (Cat. No. 560128) and analyzed on a flow cytometer. Both unstained and stained hESCs are shown in blue, negative and stained BD CompBead particles in green, and negative and stained BD CompBead Plus particles in red. Flow cytometry was performed on a BD™ LSR II flow cytometry system.

B. Lysed whole blood (LWB), BD CompBead particles, and BD CompBead Plus particles were stained with PE mouse anti-human CD3 (Cat. No. 555333) and analyzed on a flow cytometer. Stained LWB is shown in blue, negative and stained BD CompBead particles in green, and negative and stained BD CompBead Plus particles in red. Flow cytometry was performed on a BD LSR II flow cytometry system.

Beads de CAPTURA

Ordering Information

BD™ CompBead Compensation Particles

Description	Reg	Size	Cat. No.
Anti-rat Ig, κ	RUO	6 mL each	552844
Anti-rat/hamster Ig, κ	RUO	6 mL each	552845
Anti-mouse Ig, κ	RUO	6 mL each	552843

BD™ CompBead Plus Particles

Description	Reg	Size	Cat. No.
Anti-mouse Ig, κ	RUO	6 mL each	560497
Anti-rat Ig, κ	RUO	6 mL each	560499

Son Beads de Captura de un polímero plástico (2 tamaños)
Ig G kappa de ratón- anti Human o Ig G kappa de rata anti-ratón
Nos independizamos del Nivel de Expresión de la Población

Post Sorting

- **Adquisición tubos Post Sort**
- **Recuento celular**
- **Viabilidad**
- **Proliferación**

Validación del Sorting

- **Evaluar los Resultados teóricos obtenidos**
- **Verificar el recuento celular y su Pureza**
- **Decidir parámetros a modificar en futuras repeticiones**

«La última palabra la tienen las células»

Ejemplos de SORTING

BD FACS Aria II cell SORTER

Laser Azul 488 nm

Laser Rojo 633 nm

Laser Violeta 405 nm

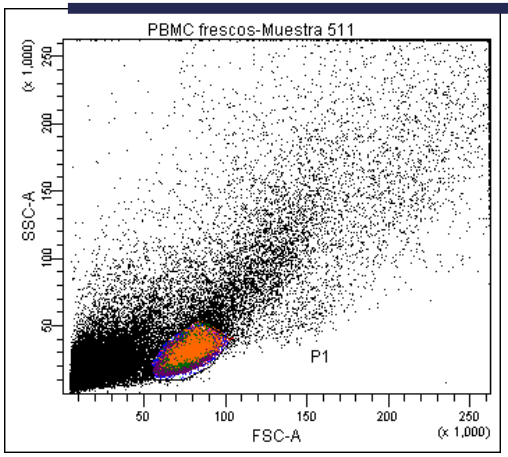
FACSDiva Software versión 6.1.3

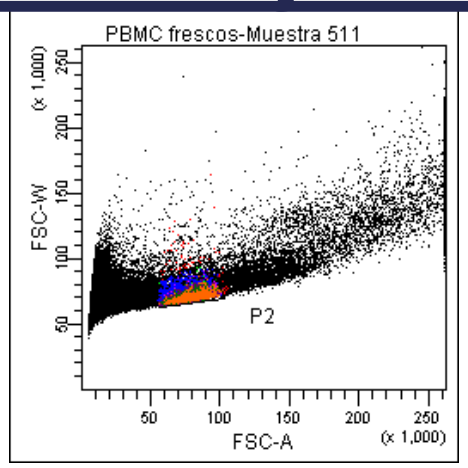
SORTING PBMCs

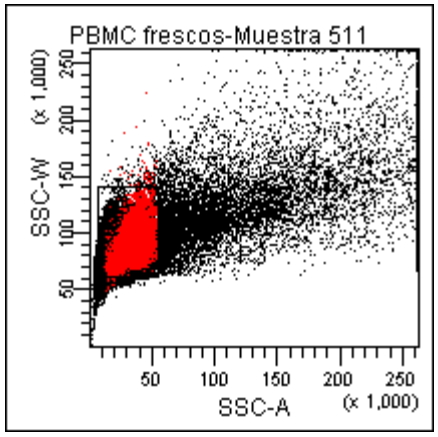
Sorting LT y NK de Sangre Periférica

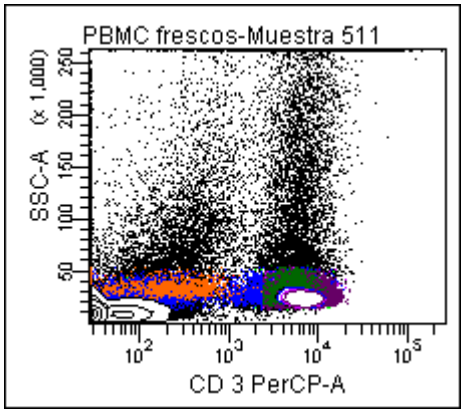
- **Muestra: Sangre entera**
- **Enriquecimiento Previo: Gradiente de Ficoll-Hypaque**
- **Temperatura del Sorting: 20 ° C**
- **Marcación: Panel 4 colores CD8 FITC**
  CD56 PE
  CD3 PerCP
  CD4 APC

SORTING de LT y NK:







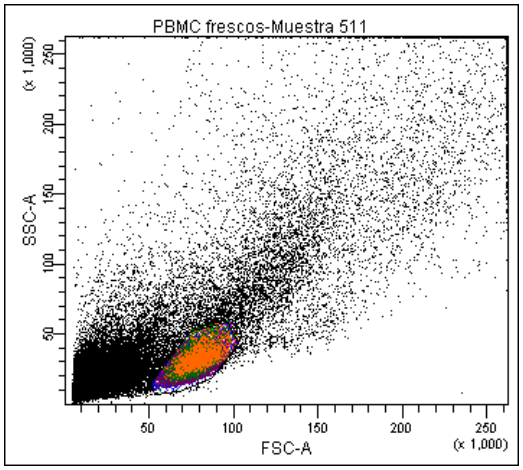


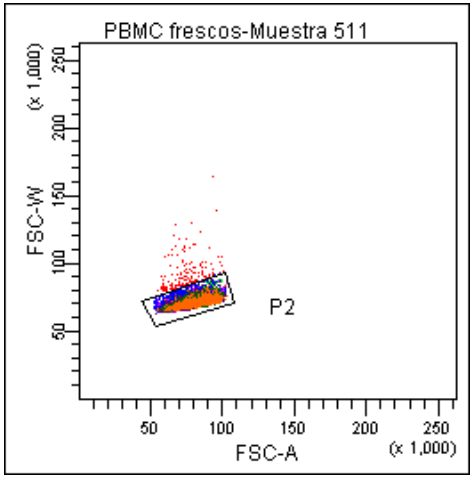
Tube: Muestra 511

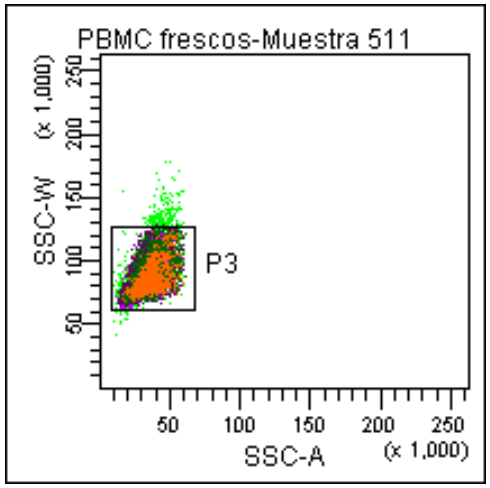
Population	#Events	%Parent	%Total
All Events	300,741	####	100.0
P1	30,648	10.2	10.2
P2	30,570	99.7	10.2
P3	30,522	99.8	10.1
LT	20,010	65.6	6.7
LTCD4+	17,557	87.7	5.8
LTCD8+	1,277	6.4	0.4
NK	1,814	5.9	0.6

Threshold + Exclusión de Dobletes + Gating

Definir Subpoblaciones:

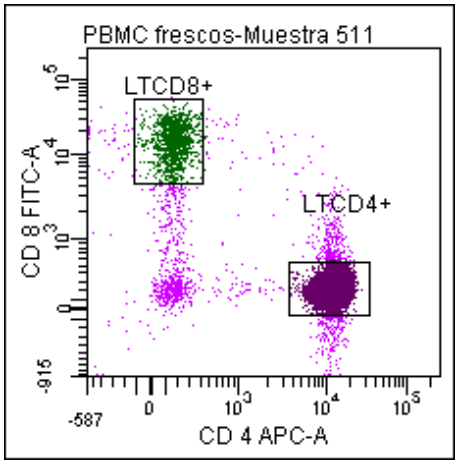


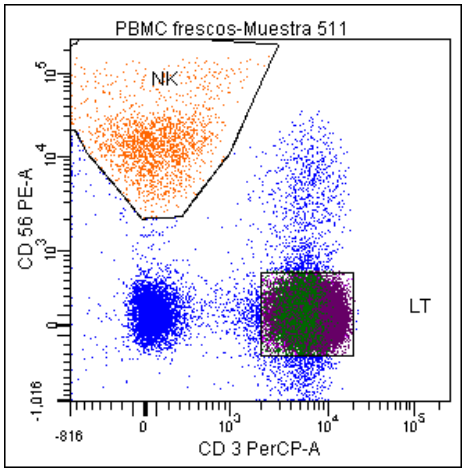




Tube: Muestra 511

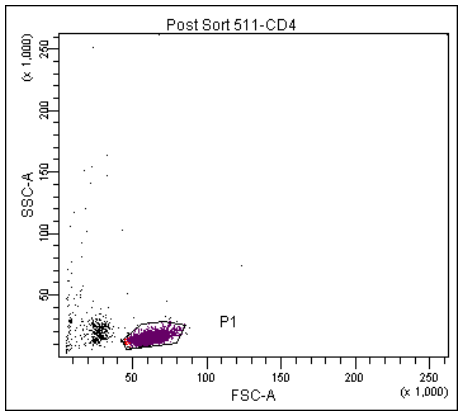
Population	#Events	%Parent	%Total
All Events	300,741	####	100.0
P1	31,036	10.3	10.3
P2	30,868	99.5	10.3
P3	30,669	99.4	10.2
LT	23,325	76.1	7.8
LTCD4+	19,141	82.1	6.4
LTCD8+	2,404	10.3	0.8
NK	1,931	6.3	0.6

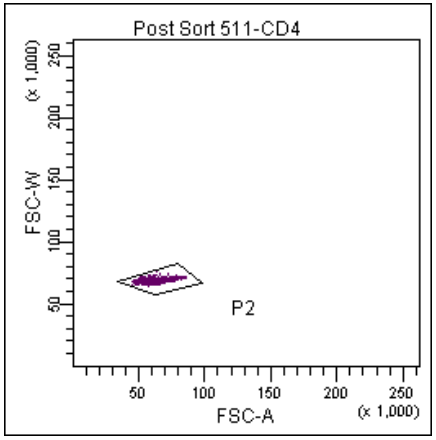


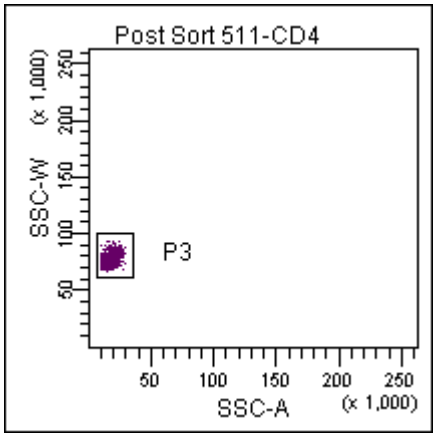


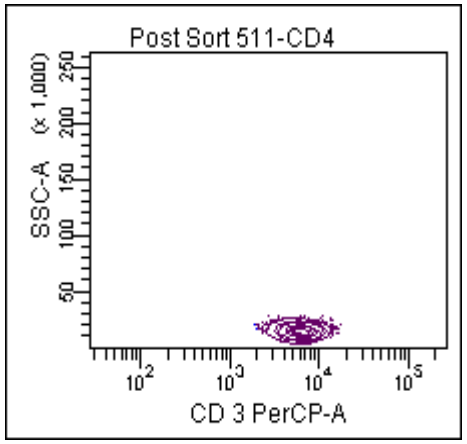
SORT Lay-Out

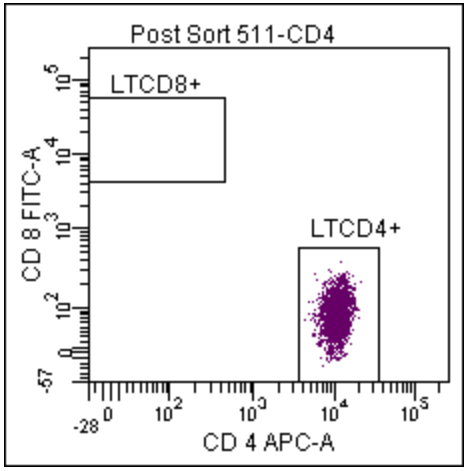
Post Sort LT CD4+







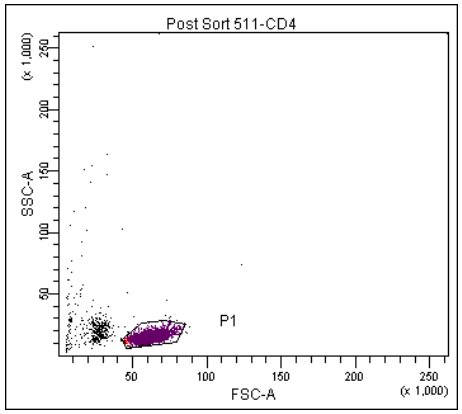


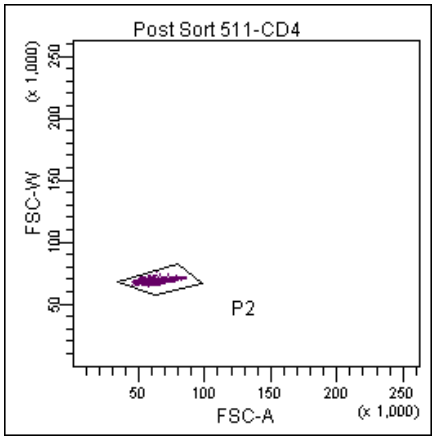


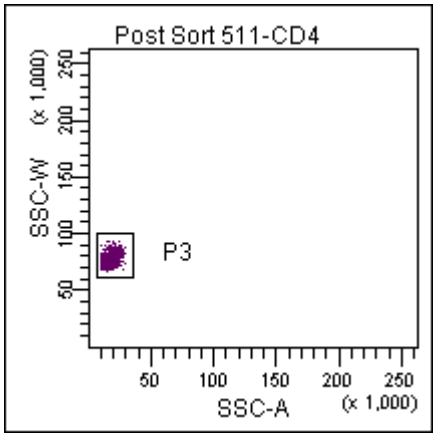
Tube: CD4

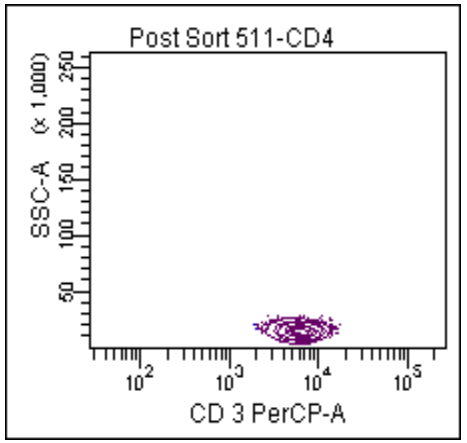
Population	#Events	%Parent	%Total
All Events	2,646	####	100.0
P1	2,173	82.1	82.1
P2	2,172	100.0	82.1
P3	2,172	100.0	82.1
LT	2,170	99.9	82.0
LTCD4+	2,170	100.0	82.0
LTCD8+	0	0.0	0.0
NK	0	0.0	0.0

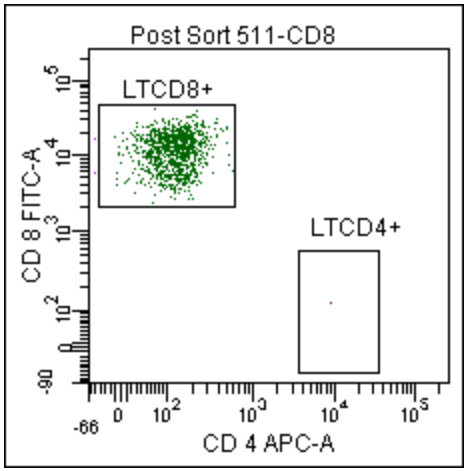
Post Sort LT CD8+







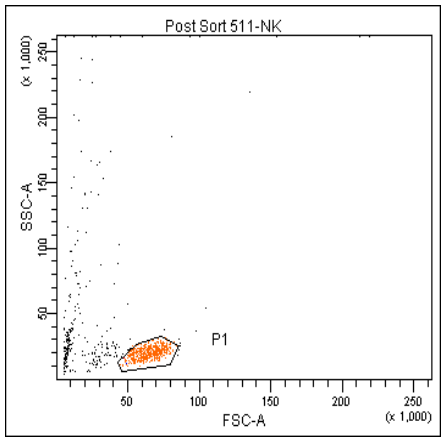


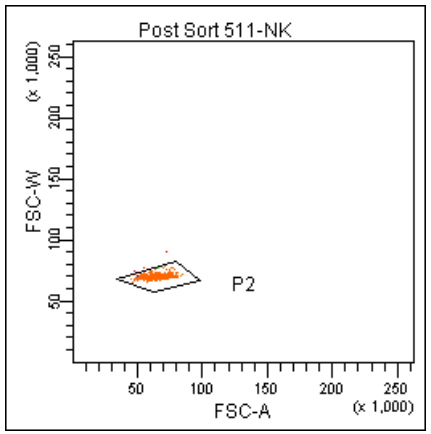


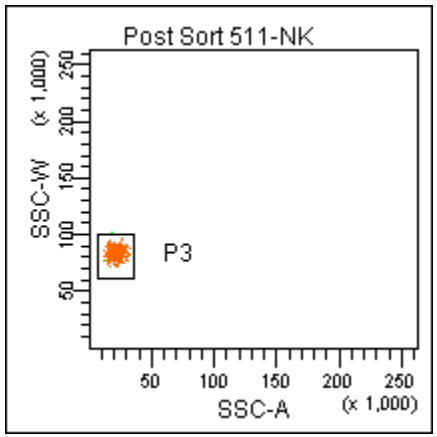
Tube: CD8

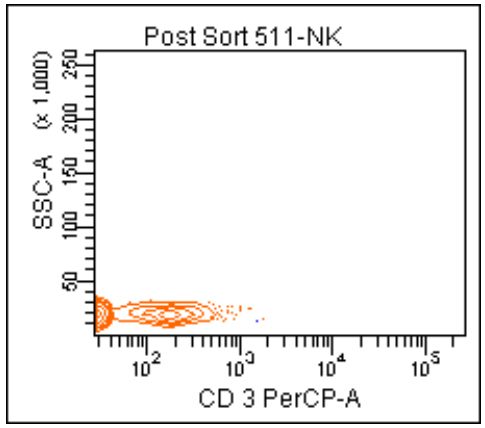
Population	#Events	%Parent	%Total
All Events	1,393	####	100.0
P1	1,012	72.6	72.6
P2	1,012	100.0	72.6
P3	1,011	99.9	72.6
LT	1,009	99.8	72.4
LTCD4+	1	0.1	0.1
LTCD8+	1,006	99.7	72.2
NK	0	0.0	0.0

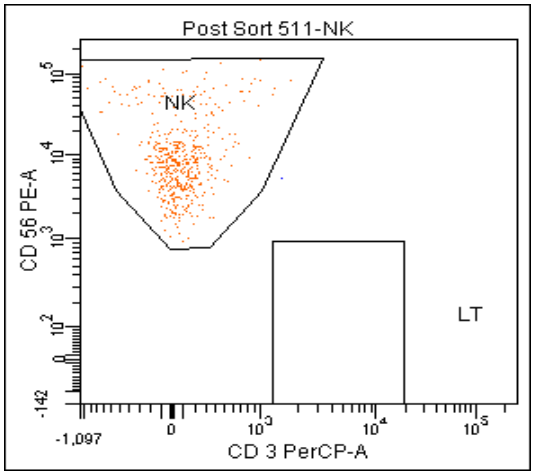
Post Sort células NK

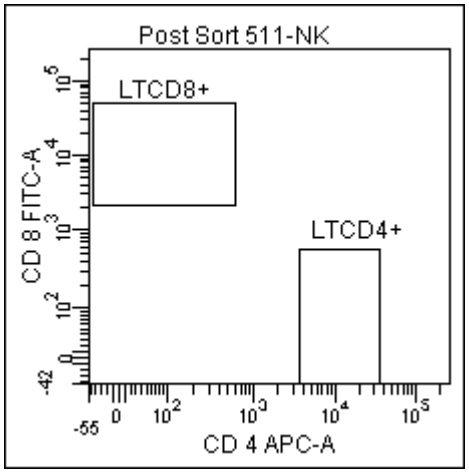












Tube: NK

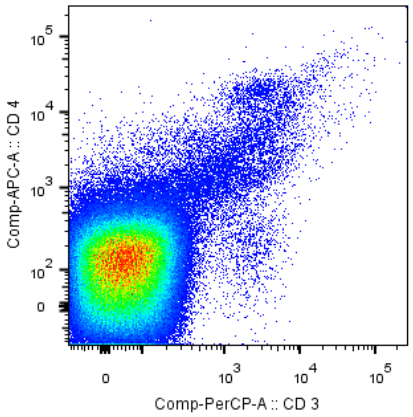
Population	#Events	%Parent	%Total
All Events	792	####	100.0
P1	489	61.7	61.7
P2	486	99.4	61.4
P3	485	99.8	61.2
LT	0	0.0	0.0
LTCD4+	0	####	0.0
LTCD8+	0	####	0.0
NK	484	99.8	61.1

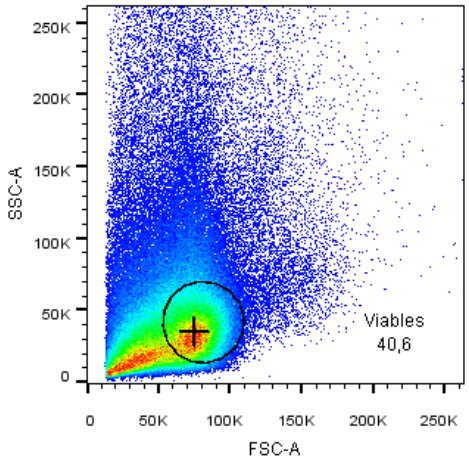
SORTING Tejido Adiposo

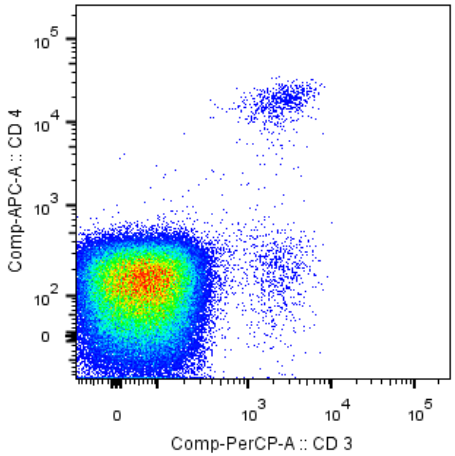
Sorting LT de Tejido Adiposo

- **Preparación de la Muestra:** Disgregación mecánica
  Colagenasa
- **Conservación: Overnight 24 hs**
- **Temperatura del Sorting: 4 ° C**
- **Sorting: 2 subpoblaciones**
- **Marcación: Panel 3 colores** CD3 PerCP
  CD4 APC
  CD25 APC-Cy7

Condiciones de Sorting



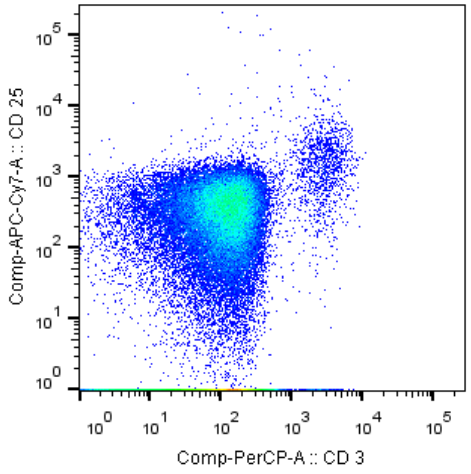


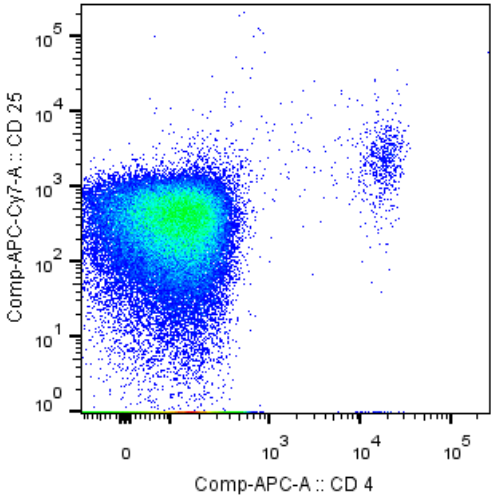


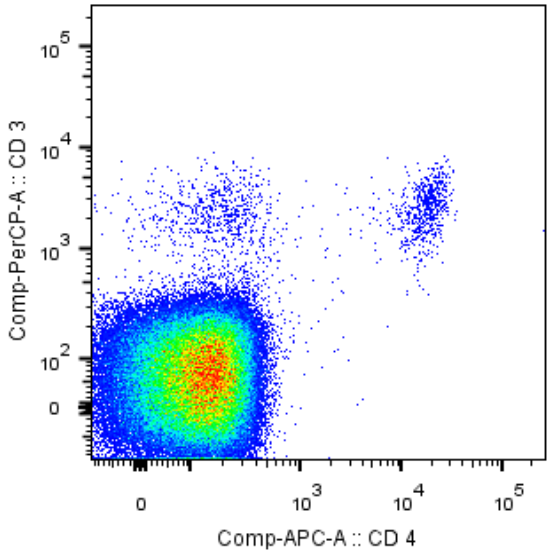
Excluimos células muertas mediante Gating

Identificamos Nubes

Tubo Pre Sort

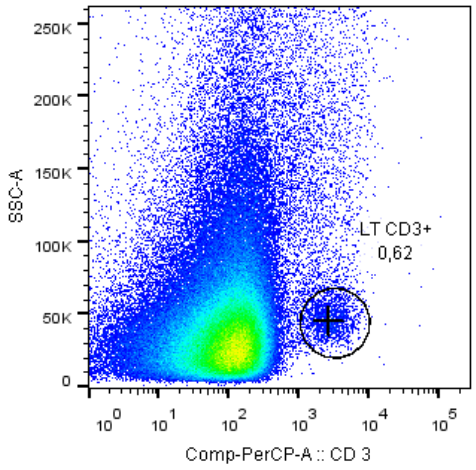


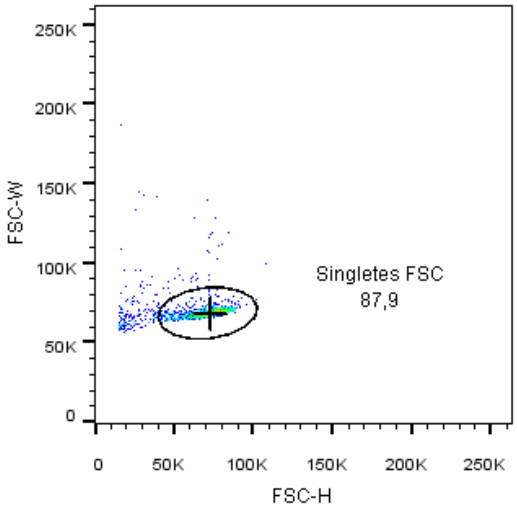


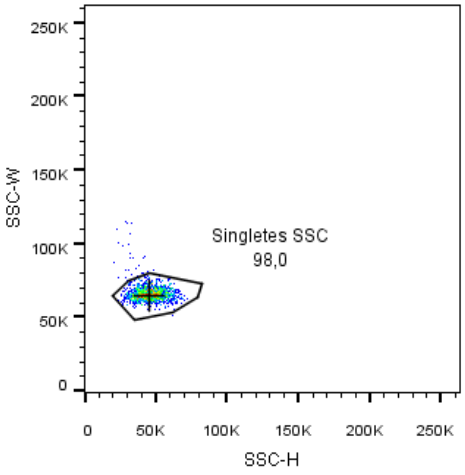


Validamos la Compensación

Criterio de Sorting

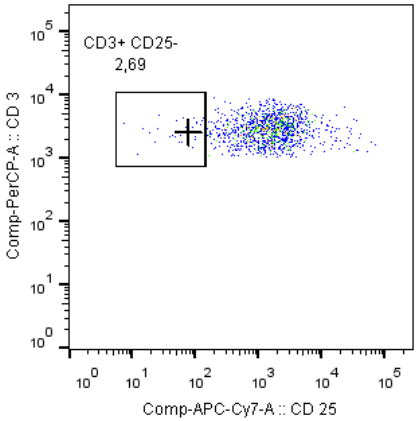


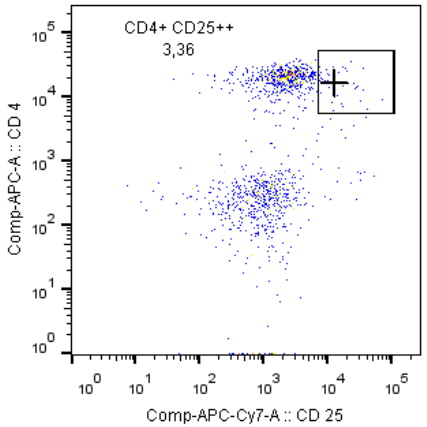




Seleccionamos Población de Interés y Excluimos Dobletes

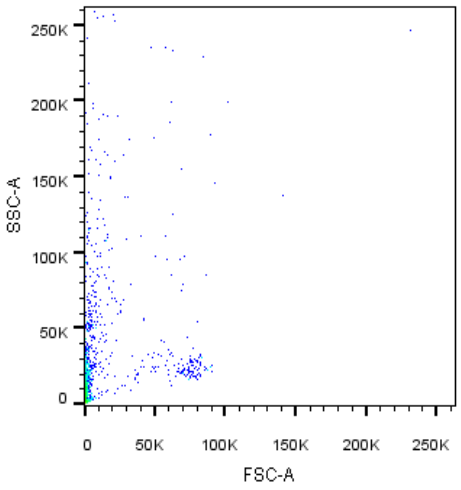
Sort Lay-Out

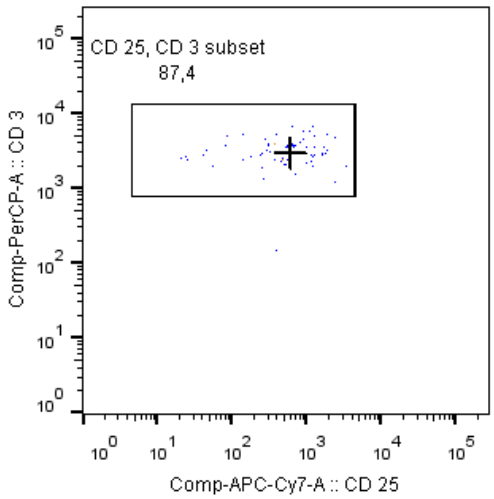


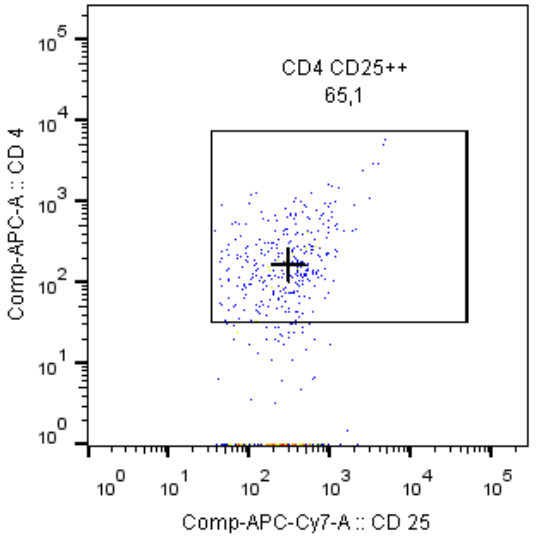


Definimos Subpoblaciones por Inmunofenotipo

Tubos Post Sort







The Journal of Immunology

This information is current as of January 28, 2019.

ATP-Induced Inflammation Drives Tissue-Resident Th17 Cells in Metabolically Unhealthy Obesity

Julieta B. Pandolfi, Ariel A. Ferraro, Inés Sananez, Maria C. Gancedo, Plácida Baz, Luis A. Billordo, Leonardo Fainboim and Lourdes Arruvito

J Immunol 2016; 196:3287-3296; Prepublished online 7 March 2016;
doi: 10.4049/jimmunol.1502506
http://www.jimmunol.org/content/196/8/3287

SORTING LT Reg CD8+

CCR5 4 Subpoblaciones

SORTING Subpoblaciones LT CD8+

- **Muestra**: Sangre entera

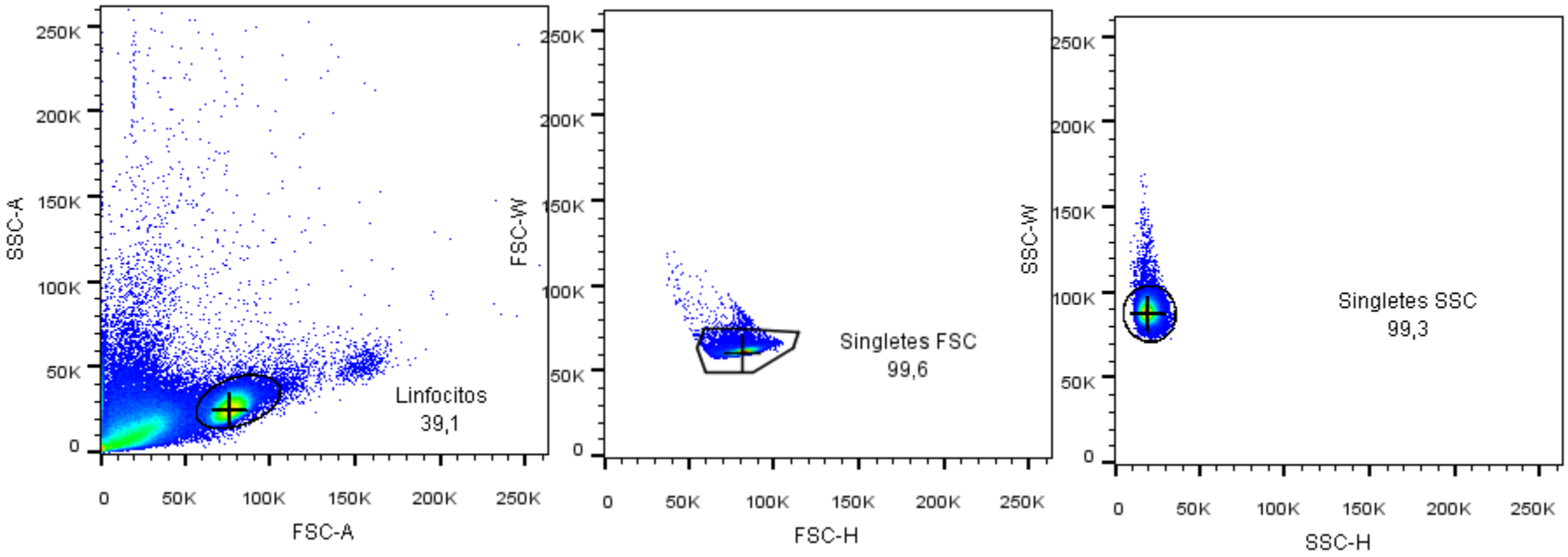
- **Sorting**: 4 subpoblaciones

- **Marcación**: Panel 3 colores: HLA-DR PE
  CCR5 PE-Cy7
  CD8 APC

- **Enriquecimiento previo**:

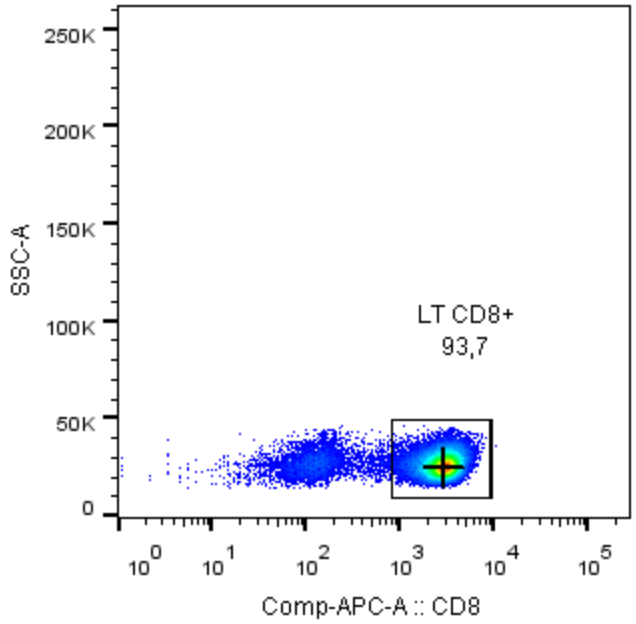
1) Gradiente Ficoll-Hypaque (MNT)
2) Rosetas T: Selección Negativa LT CD8 +
   (RosetteSep ®Stem cell technologies)

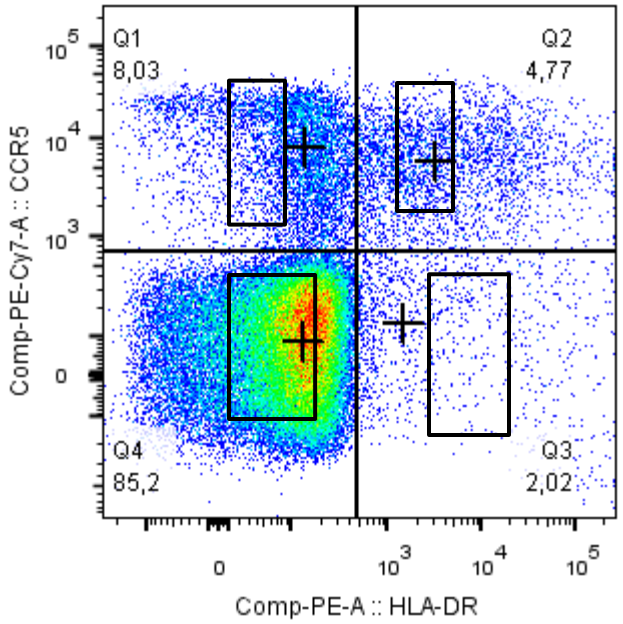
Pre Sort



Gating Linfocitos y Exclusión de Dobletes

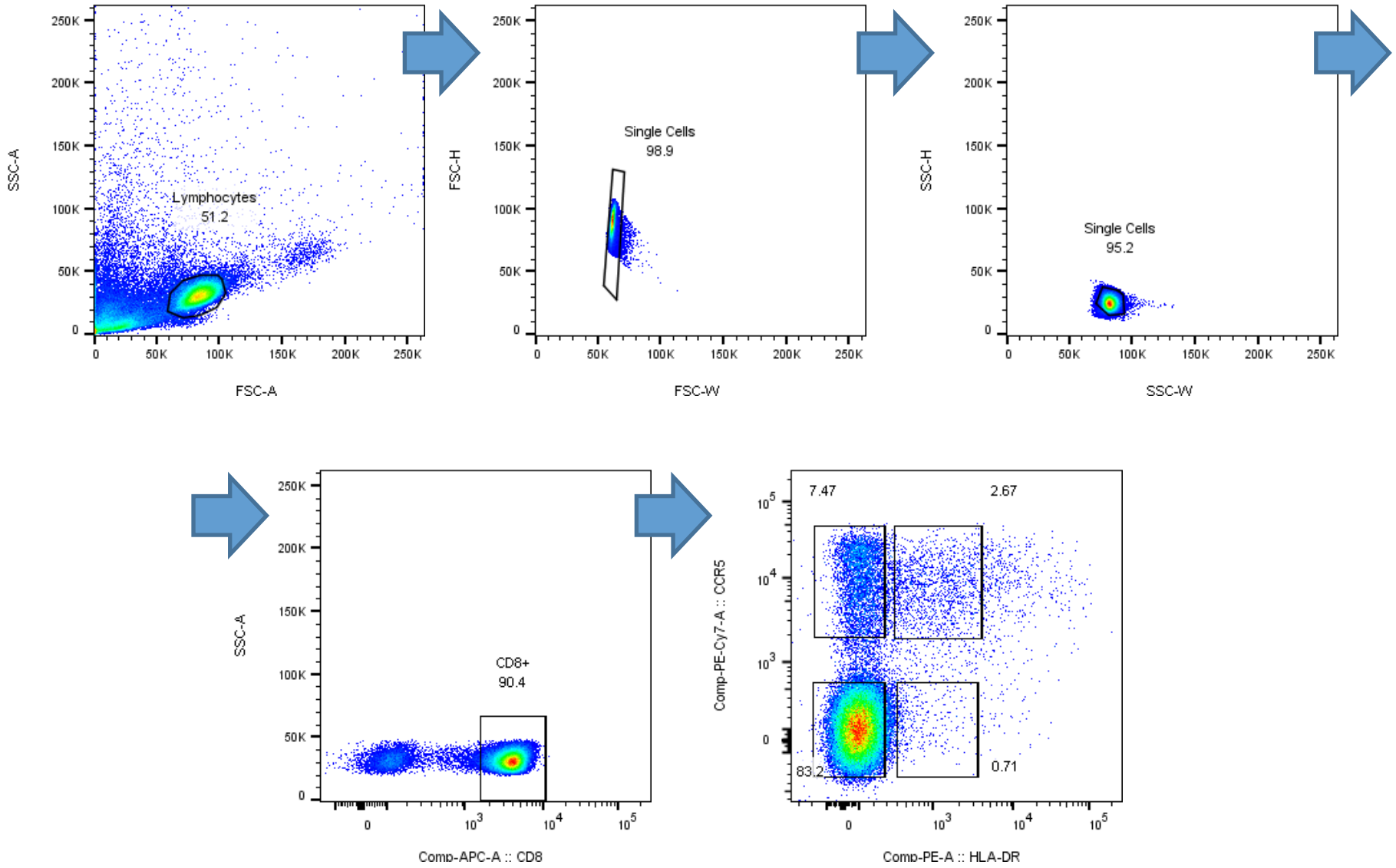
Criterio de Sorting



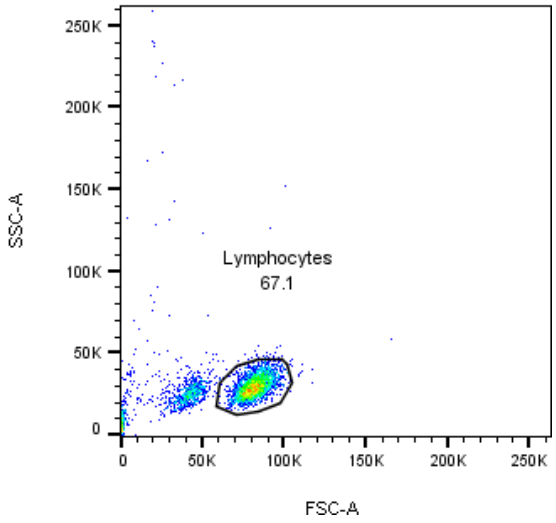


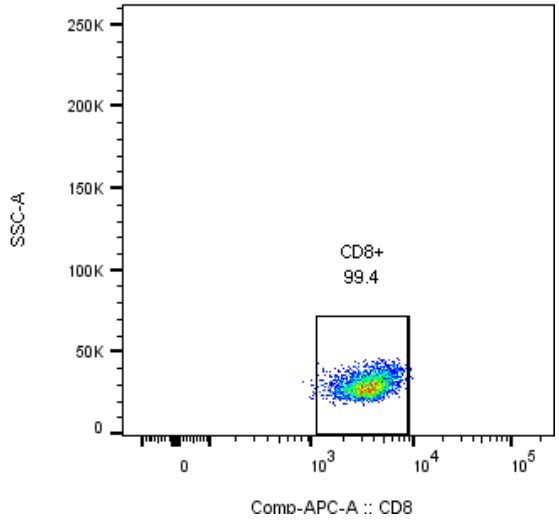
Definimos Subpoblaciones por Inmunofenotipo

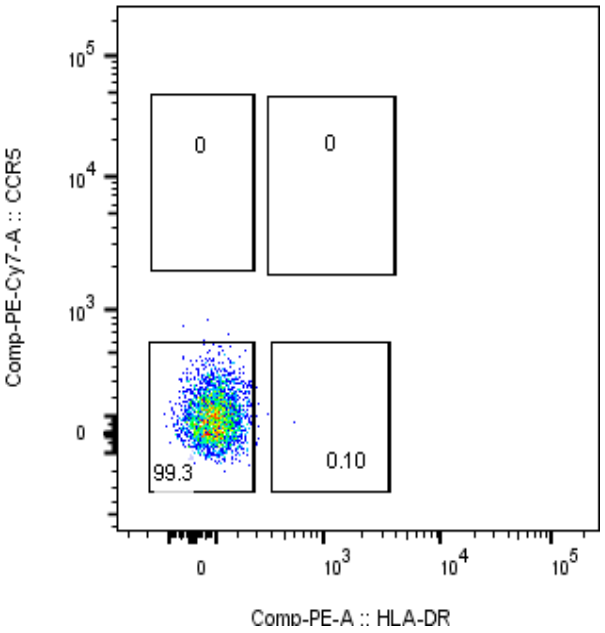
Tubo Pre-sort: 4 subpoblaciones

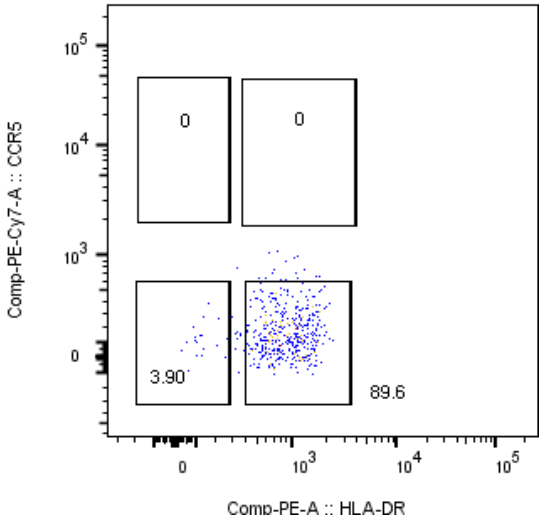


Tubos Post Sort





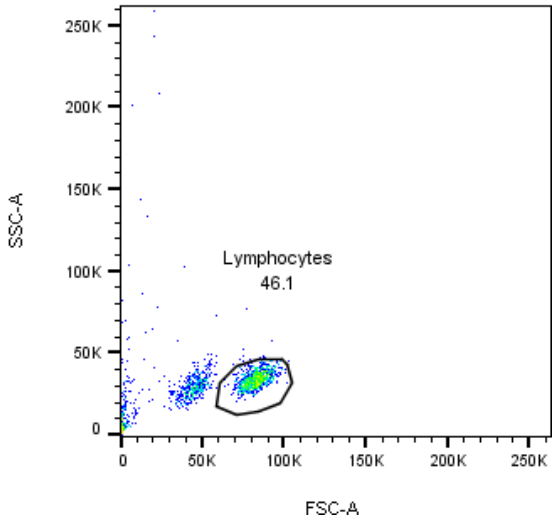


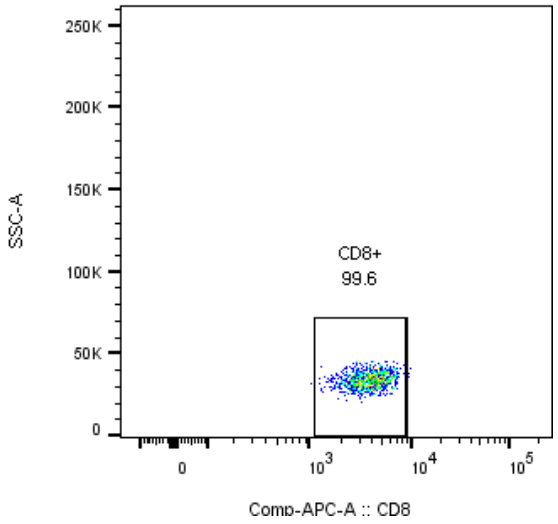


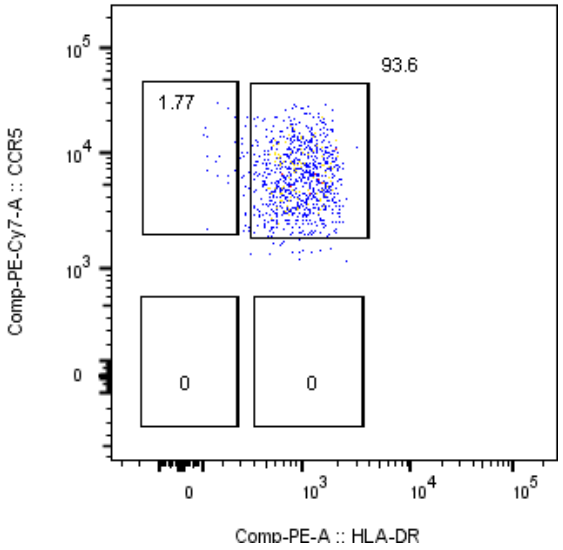
DR- CCR5-

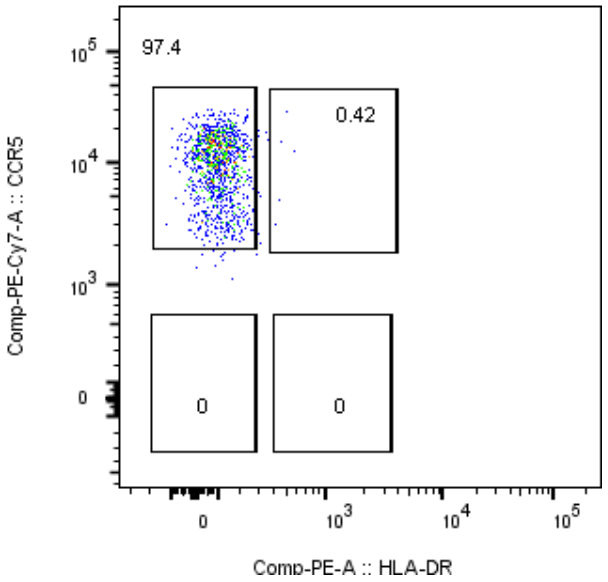
DR+ CCR5-

Tubos Post Sort









DR+ CCR5+

DR- CCR5+

frontiers in Immunology

ORIGINAL RESEARCH
published: 29 November 2018
doi: 10.3389/fimmu.2018.02789

Human CD8$^+$HLA-DR$^+$ Regulatory T Cells, Similarly to Classical CD4$^+$Foxp3$^+$ Cells, Suppress Immune Responses via PD-1/PD-L1 Axis

*Andres Machicote[1], Santiago Belén[1], Placida Baz[1], Luis A. Billordo[1] and Leonardo Fainboim[1,2]**

SORTING MM

Muestra: MO

Sorting células plasmáticas patológicas

- **Se separó por el método de cell-Sorting efectuado a partir de una muestra de Médula ósea**
- **Citómetro uFACSAria II con tres lasers (violeta: 405nm, azul: 488 nm, rojo:633 nm) y configuración para detectar hasta 13 colores**
- **Inmunomarcación de superficie con Panel cuatro colores**
- **Se seleccionó la población a separar por Inmunofenotipo las Células Plasmáticas Patológicas (CPP) con exclusión de dobletes y células muertas**

Separación CPP de MO

- **Inmunofenotipo:**

  CD45 - / CD19 - / CD38 + / CD138 +

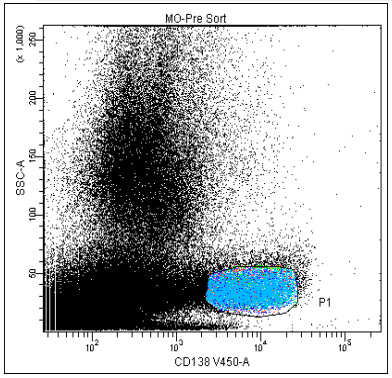
- **Marcación 4 colores:**

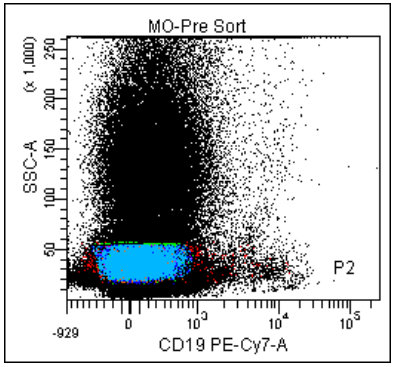
  PerCP / PE-Cy7 / APC-H7/ V450

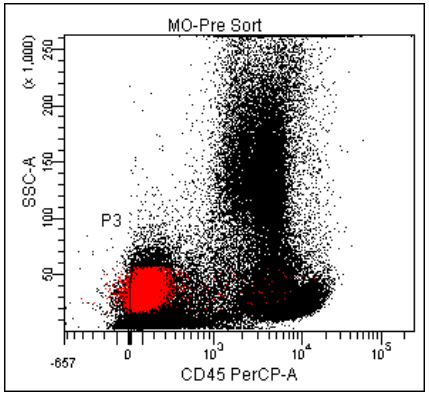
- **Compensación: Automática (COMPBeads)**

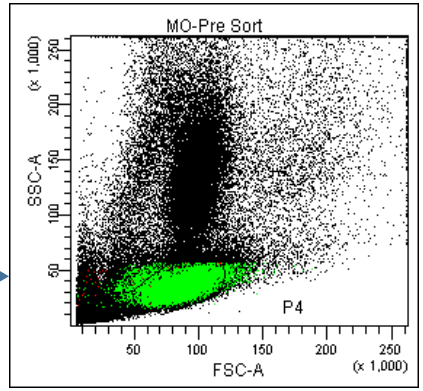
- **Medio de recolección: MeOH : AcH (3:1)**

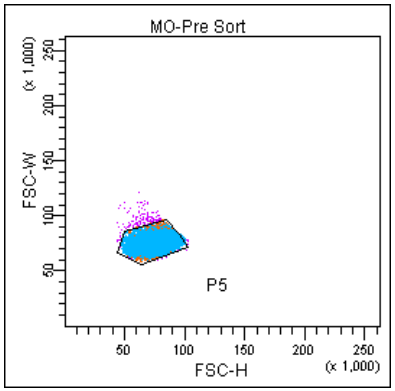
Criterio de Sort: tubo Pre Sort

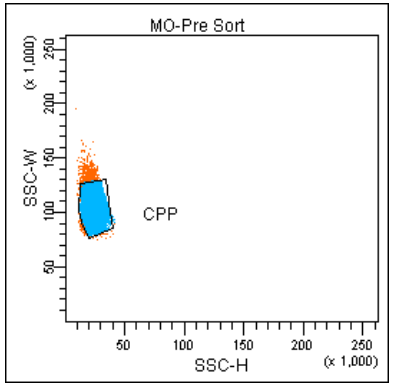












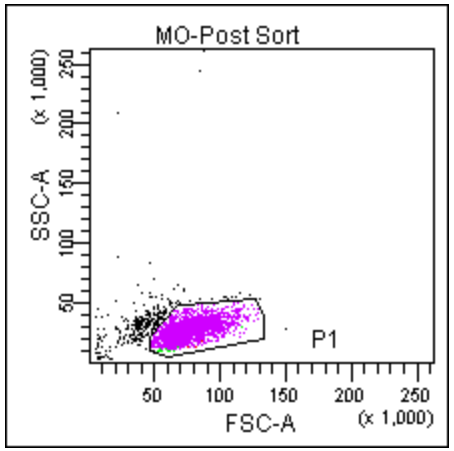
Tube: Pre Sort

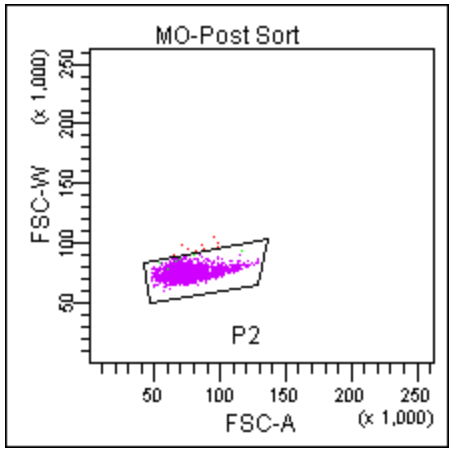
Population	#Events	%Parent	%Total
All Events	462,834	####	100.0
P1	155,058	33.5	33.5
P2	154,478	99.6	33.4
P3	151,093	97.8	32.6
P4	131,541	87.1	28.4

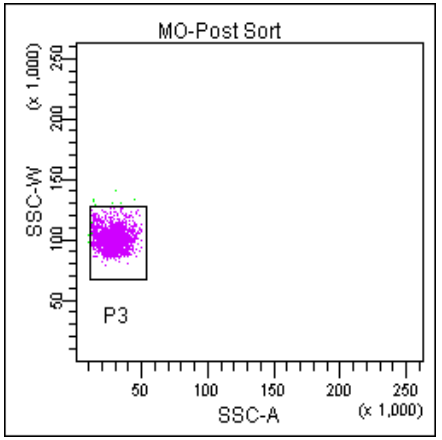
CPP

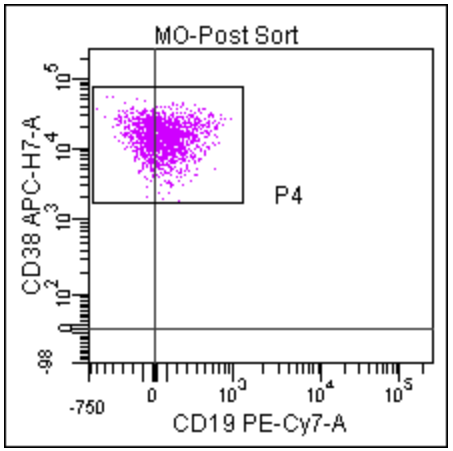
Exclusión de Dobletes

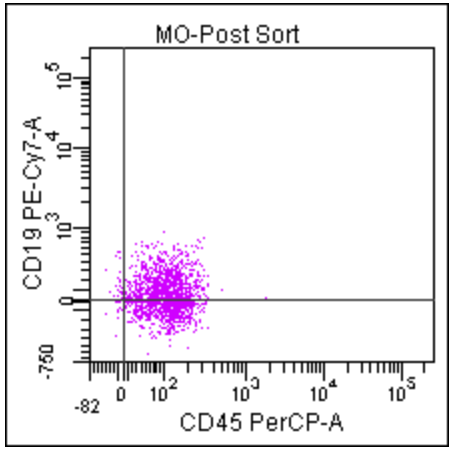
Resultados obtenidos: Post Sort

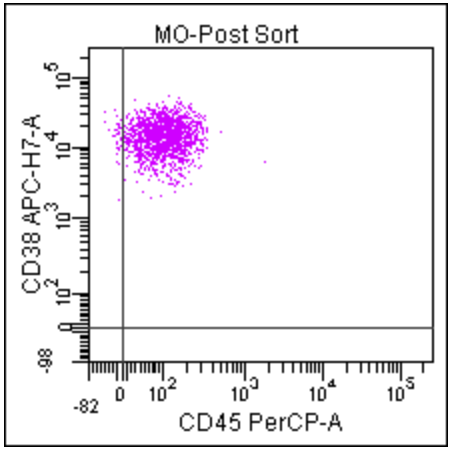


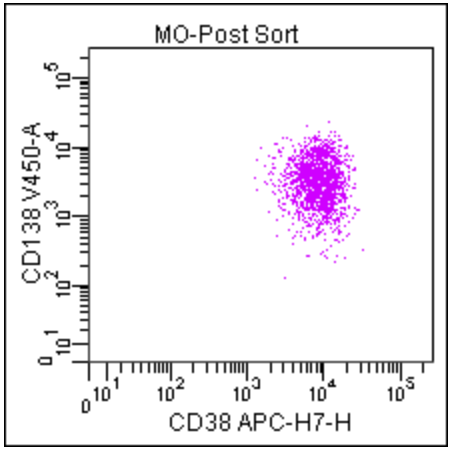












Tube: Post Sort

Population	#Events	%Parent	%Total
All Events	2,024	####	100.0
P1	1,687	83.3	83.3
P2	1,677	99.4	82.9
P3	1,655	98.7	81.8
P4	1,635	98.8	80.8

CPP: 71307 (98.8 %)

Condiciones del Sorting

Experiment : SORTING MM 280218	Sort Report	Report Date : 2018.02.28 at 16:43:28
Specimen : MO		Device : 4 Tube
Tube : Global Sheet1		User ID : Administrator
Sort Layout : Sort Layout 280218		Cytometer : FACSAriaII (1)
Application : FACSDiva Version 6.1.3		

Sort Settings

Sort Setup	70 micron	Phase Mask	0
Frequency	88.3	Single Cell	Off
Amplitude	13.2	Sweet Spot	On
Phase	0.00	First Drop	141
Drop Delay	42.49	Target Gap	6
Attenuation	Off	Plates Voltage	4,500
Precision	Purity	Voltage Centering	0
Yield Mask	32	Sheath Pressure	70.00
Purity Mask	32		

Side Stream Voltage (%)

Far Left	Left	Right	Far Right
100.00	42.00	40.00	100.00

Neighboring Drop Charge (%)

2nd	3rd	4th
17.00	8.00	0.00

Acquisition Counters

Threshold Count	2743880
Processed Events Count(evt)	854965
Electronic Aborts Count(evt)	7615
Sort Elapsed Time(hh:mm:ss)	00:55:51

Sort Counters

	Far Left	Left	Right	Far Right
Sort Rate(evt/s)	NA	NA	21	NA
Conflicts Count(evt)	NA	NA	1431	NA
Conflicts Rate(evt/s)	NA	NA	0	NA
Efficiency(%)	NA	NA	98	NA

Sort Layout

Far Left	Left	Right	Far Right
		CPP : 71307	

FISH

- **Se identificaron en el servicio de Citogenética por la técnica de FISH las mutaciones correspondientes**
- **Se estudian 4 translocaciones**
- **Se analizan 300 núcleos para cada una ellas**

Conclusiones

- **La Citometría de Flujo nos permite la identificación y selección multiparamétrica de subpoblaciones .**

- **Mediante cell SORTING podemos separarlas con eficiencia.**

- **Se obtienen diversos tipos celulares con alta pureza.**

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  Delynn Moss and Stephen Prefetto . Special Report CDC January 2007

¡ Muchas gracias !

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